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Spectroscopic labeling of A, S/T in the $^{1}H–^{15}N$ HSQC spectrum of uniformly ($^{15}N–^{13}C$) labeled proteins

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ABSTRACT

A new triple resonance two-dimensional experiment, termed (HC)NH, has been described to generate specific labels on the peaks of alanines and serines/threonines, separately, in the $^{1}H–^{15}N$ HSQC spectrum of a protein. The performance of the pulse sequence has been demonstrated with a 151 residue protein. The method permits the investigation of local environments around those specific residues without actually having to obtain complete resonance assignments for the entire protein. With this one can envisage use of the technique for studying large protein systems from different points of view.

1. Introduction

The development of multi-dimensional triple resonance NMR experiments has been a major breakthrough in the structure elucidation of proteins in aqueous solutions [1,2]. However, the complete backbone assignment followed by NMR structure calculation may be a cumbersome job for a biologist or a chemist interested to monitor the changes in a given protein under different experimental conditions at certain specific residues. The partial answer to this problem has been through the development of specific labeling schemes [3], wherein peaks corresponding to certain specifically labeled amino acids are obtained in the HSQC spectrum, and assignment can be obtained by preparing different mutants of the system. For example, if it is required to study only alanines in a protein, various point-mutants of the alanine labeled protein can be prepared wherein different alanines have been mutated/deleted in different mutants, and HSQC spectra of the different mutant proteins can be separately recorded. In this way, one can assign all the alanines in the protein without going through the laborious procedure of complete backbone resonance assignment. However, these selective labeling schemes suffer from various disadvantages. The first and the major problem associated with selective labeling is 'scrambling' of various amino acids labels. For example, the labeling of alanine residues scrambles the label to valines and leucines side-chains. Likewise, the labeling of serine in the protein also labels glycines and cysteins while an attempt to label threonines also labels the isoleucines. In addition to this problem, the use of specifically labeled amino acids in the bacterial growth media as compared to uniformly labeled $^{15}NH_4Cl$ and ^{13}C-glucose raises the cost per sample by many folds and seems to be an uneconomical affair.

Spectroscopic labeling of selected residues in an HSQC spectrum provides a useful alternative. Tugarinov et al. suggested a pulse sequence for labeling alanines in a large 723 residue protein [4]. Alternative schemes based on 2D versions of other pulse sequences such as HNN, HN(C)N, HN(CO)CA, etc. can be conceived. In this background, we present here a simple two-dimensional triple resonance NMR pulse sequence that works on a uniformly (^{15}N, ^{13}C) labeled protein. The pulse sequence, designated as (HC)NH, can be tuned to generate alanine or serine–threonine labels in the HSQC spectrum. The ideas used here have been derived from an application described by us recently [5–7] in the context of three-dimensional HNN suite of experiments. However, the pulse sequence employs a smaller number of transfer steps and has fewer constant time periods, and thus has a higher sensitivity compared to what one could have derived from the HNN suite of experiments or from the one described by Tugarinov et al. [4]. The performance of the (HC)NH experiment has been demonstrated using a 151 residue protein, *Eh*CaM (calmodulin-like Ca^{2+}-binding protein from the protozoan parasite *Entamoeba histolytica*).

2. Results and discussion

2.1. The (HC)NH pulse sequence

The new pulse sequence (HC)NH (Fig. 1) employs the following magnetization transfer pathway:

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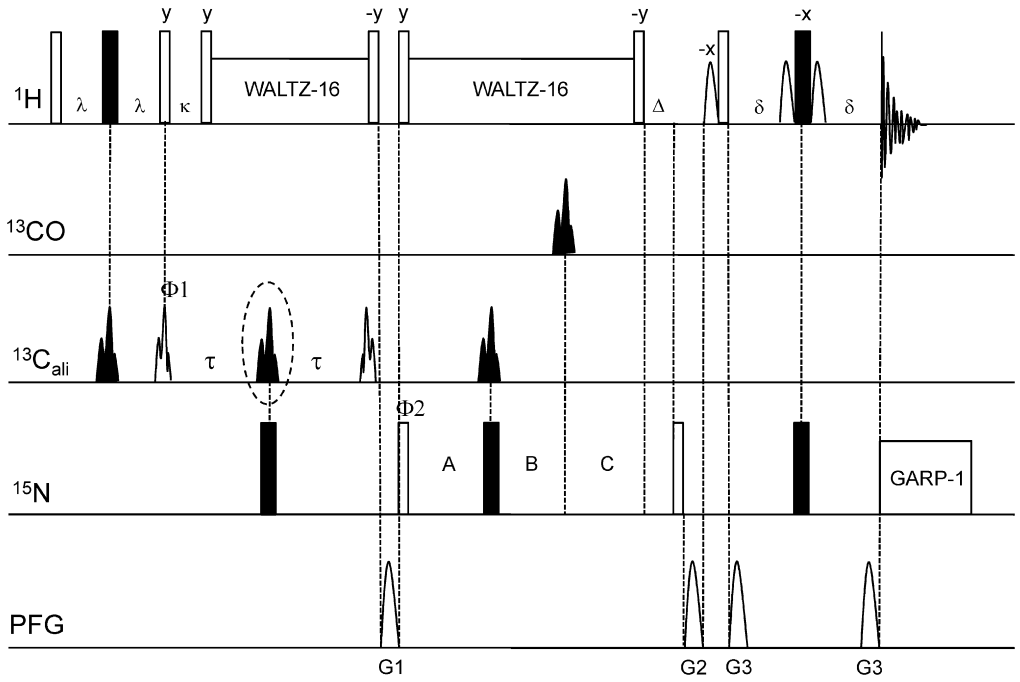

Fig. 1. The pulse sequence for the (HC)NH experiment. Narrow (hollow) and wide (filled black) rectangular bars represent non-selective 90° and 180° pulse, respectively. Hollow and black filled lobes on carbon channel indicate selective 90° and 180° pulse, respectively. Unless indicated otherwise, the pulses are applied with phase *x*. The ^{1}H and ^{15}N carrier frequencies are set at 4.7 ppm (water) and 116.0 ppm, respectively. The ^{13}C carrier frequency is set at 56.0 ppm, except for the encircled pulse in case of serine–threonine selective experiment, where it is set at 64.0 ppm. Proton decoupling using the Waltz-16 decoupling sequence [14,15] with field strength 6.3 kHz is applied during the 2τ and $2T_N$ periods, and ^{15}N decoupling using the Garp-1 sequence [16] with field strength 0.9 kHz is applied during acquisition. The strength of $^{13}C^{\alpha}$ pulses (standard Gaussian cascade Q3 pulse) [13] has been adjusted to cause minimal excitation of carbonyl carbons. The band-selective 180° pulse in the experiment—responsible for alanine selection or serine–threonine selection—has been encircled for emphasis. The 180° ^{13}CO shaped pulse (width 0.6 ms) had a standard Gaussian cascade Q3 pulse profile with minimum excitation of $^{13}C^{\alpha}$. The delays are set to $\lambda = 1.7$ ms, $\kappa = 3.6$ ms, $\Delta = 5.4$ ms, and $\delta = 2.7$ ms. T must be optimized and is around 12–16 ms. The values for individual periods containing t_1 are: $A = t_1/2$, $B = T_N$, and $C = T_N - t_1/2$. Phase cycling for the experiment is $\Phi1 = x,-x$; $\Phi2 = x,x,-x,-x$ and receiver = $x,-x,-x,x$. Frequency discrimination in t_1 has been achieved using State-TPPI phase cycling of $\Phi2$ along with the receiver phase. The gradient (sine-bell shaped; 1 ms) levels are as follows: $G1 = 30\%$, $G2 = 30\%$, and $G3 = 80\%$ of the maximum gradient strength 53 G/cm in the *z*-direction.

$$H_i^{\alpha} \rightarrow C_i^{\alpha} \xrightarrow{2\tau} N_{i,i+1}(t_1) \xrightarrow{2T_N} H_{i,i+1}(t_2) \tag{1}$$

The magnetization is first transferred from the H^{α} atom of the *i*th residue to the C^{α} atom of the *i*th residue. The C^{α} magnetization is allowed to evolve for the period 2τ under $^1J_{C^{\alpha}-N}$ and $^2J_{C^{\alpha}-N}$ coupling and then transferred to backbone nitrogens of *i*th and $i+1$-th residues. During the next $2T_N$ period the nitrogens get frequency labeled (t_1 evolution) and nitrogen magnetization that is anti-phase to carbon is refocused. The magnetization is then finally transferred to H^N atom of *i*th and $i+1$-th residue and detected, thus resulting in two peaks per residue, one self and one sequential, in the 2D spectrum at the following coordinates:

$$(F_2, F_1) = (H_i, N_i), (H_{i+1}, N_{i+1}) \tag{2}$$

Thus, starting from each residue *i* one generates two peaks at the HSQC coordinates of *i* and $i+1$ residues. For example, in a tri-peptide XYZ, the sequential peak originating from H^{α} atom of X residue would overlap with the self-peak which originates from H^{α} atom of Y residue because of degenerate chemical shifts. Similarly, the sequential peak originating from H^{α} atom of Y residue would overlap with the self-peak which originates from H^{α} atom of Z residue. Thus, effectively the spectrum would be similar to normal HSQC spectrum displaying one peak per residue. But the intensity of each peak will have two contributions. This has been depicted schematically in Fig. 3A. The pulse sequence in Fig. 1 employs the WATERGATE [8] sequence for water suppression before detection and frequency selection along the indirect dimension is achieved by the States-TPPI method [9].

The intensities of the self (I_i) and the sequential (I_{i+1}) peaks for each residue have been calculated using the product operator formalism [10] and are given by Eqs. (3) and (4), respectively:

$$I_i = E_1 E_2 E_3 K_i \tag{3}$$

$$I_{i+1} = E_1 E_4 E_5 K_{i+1} \tag{4}$$

where

$$\begin{aligned} E_1 &= \cos n_i\tau \\ E_2 &= \cos q_i\tau \sin p_i\tau \\ E_3 &= \cos q_{i-1}T_N \sin p_i T_N \\ E_4 &= \cos p_i\tau \sin q_i\tau \\ E_5 &= \cos p_{i+1}T_N \sin q_i T_N \end{aligned} \tag{5}$$

$$\begin{aligned} K_i &= \exp\left[-2\tau R_{2,i}^{C^{\alpha}} - 2T_N R_{2,i}^{N}\right], \\ K_{i+1} &= \exp\left[-2\tau R_{2,i}^{C^{\alpha}} - 2T_N R_{2,i+1}^{N}\right], \end{aligned} \tag{6}$$

and

$$\begin{aligned} p_i &= 2\pi {}^1J(C_i^{\alpha} - N_i), \quad q_i = 2\pi {}^1J(C_i^{\alpha} - N_{i+1}), \\ n_i &= 2\pi {}^1J(C_i^{\alpha} - C_i^{\beta}) \end{aligned} \tag{7}$$

where ^1J and ^2J correspond to one-bond and two-bond coupling constants, respectively, and the R_2s correspond to the various transverse relaxation rates.

Fig. 2 shows plots of the peak intensities vs. the time periods τ and T_N. For best transfer of magnetization, the 2τ period should be chosen to be between 22 and 30 ms and the $2T_N$ period to be between 24 and 32 ms. A detailed study of the above equations reveals that the term E_1 is negative for the optimum choice of transfer period ($\tau = 12$–14 ms) whereas the terms E_2 to E_5 remain positive (Eq. (5)). This results in negative intensities for the self as well as sequential peaks (Eqs. (3) and (4)), however, the spec-

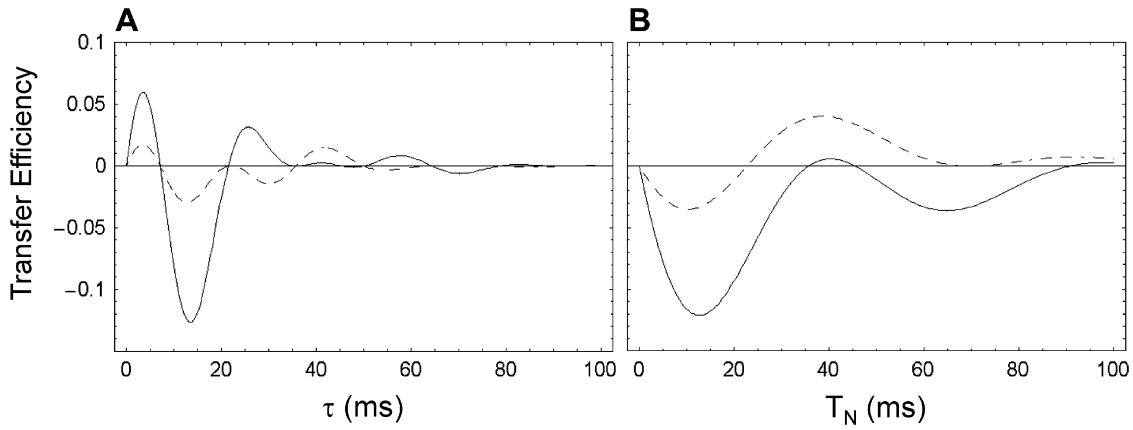

Fig. 2. Plots of the (HC)NH coherence transfer efficiencies (A) with respect to variable τ, and (B) with respect to variable T_N. The transfer functions for the diagonal (solid line in the figure) and the sequential (dashed line in the figure) are described in the text (Eqs. (3) and (4)). The plots were calculated by using $^1J_{C^\alpha-C^\beta}$, $^1J_{C^\alpha-N}$, and $^2J_{C^\alpha-N}$ values of 35, 11, and 7 Hz, respectively. The values of transverse relaxation terms $R_2(C^\alpha)$ and $R_2(N)$ used here are 30 and 15 s^{-1}, respectively. In (A), the value of T_N is 15 ms, and in (B), value of τ is 12 ms. The occurrence of minima in both the curves at a value close to τ = 12–16 ms in (A), and at T_N = 12–16 ms in (B) is indicative of maximum transfer efficiency (I_i and I_{i+1} are overall negative, Eqs. (3) and (4)) around these values. The difference in transfer efficiency in dashed and solid line represents the difference in intensity of self and sequential peaks.

trum can easily be phase shifted by 180° in the $F_2(H^N)$ dimension, to match it with the normal HSQC spectrum. The terms E_2 and E_3 are always greater than E_4 and E_5, respectively, thus making I_i greater than I_{i+1} (Eqs. (3) and (4)), and this is depicted by the size of the circles in Fig. 3.

Let us now consider a case where C^α–C^β coupling evolution does not occur for a particular residue, say, Y (in the tri-peptide XYZ). Consequently, the E_1 term for that residue will be unity (Eq. (5)) and thus the self and the sequential peaks for Y residue would be oppositely phased with respect to those of X and Z residues; if X and Z are phased to be positive, then the Y peak would be negative. Therefore, when the self-peak of Y residue overlaps with the sequential peak of X residue, there is a cancellation of intensities rather than co-addition, and since the self-peak has always higher intensity than the sequential peak, the resultant sign of the peak in the HSQC spectrum would be that of the self-peak. Thus, the peak would appear with an overall negative intensity. The Y residue would, therefore, be identified straight-away in the HSQC spectrum from the opposite sign of the peak with respect to that of the rest of the residues.

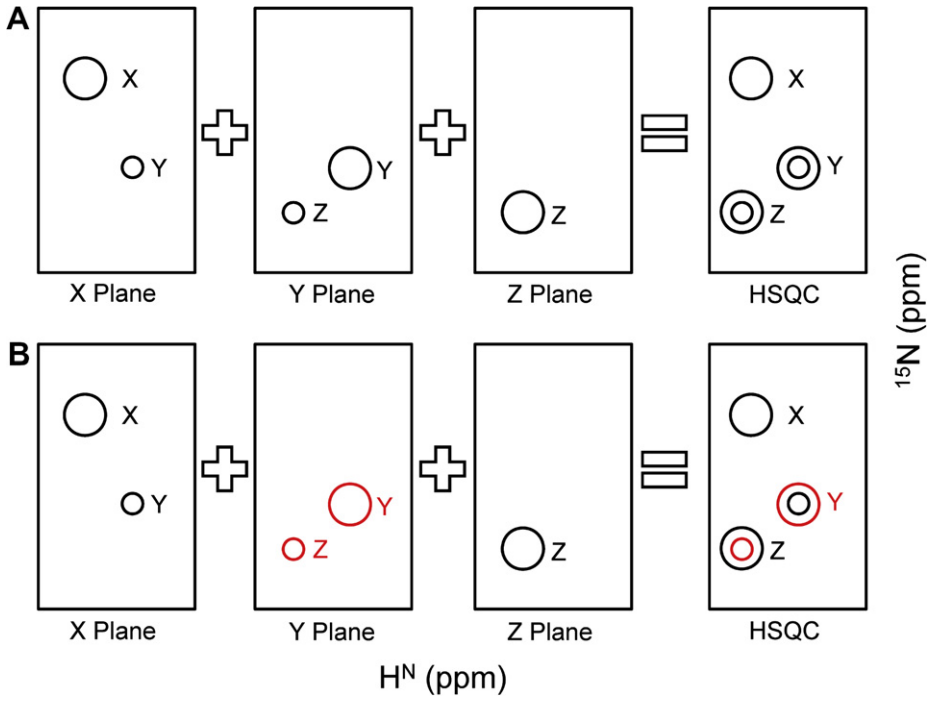

Fig. 3. (A) Schematic to explain how an HSQC spectrum for a tri-peptide, XYZ, is obtained as a result of the (HC)NH pulse sequence: In a hypothetical three-dimensional experiment where every residue is put on a separate plane and carries the information of the (HC)NH pulse sequence, every residue (except Z) in its plane contains a set of two peaks as indicated by two circles; Z residue does not have any $i+1$ residue and thus Z residue plane contains only one self-peak. Size of the circle is indicative of difference in intensity of self and sequential peaks. In the actual 2D (HC)NH, we observe superposition of these planes, giving a final spectrum similar to what is obtained in an HSQC experiment. Every peak would be a summation of two peaks—self-peak from the ith plane and sequential peak from the $i-1$-th plane. (B) A special case where no C^α–C^β coupling evolution takes place for residue 'Y' and thus the peaks corresponding to that plane are negative in intensity (shown in red). The final sign of the peak upon summation of all peaks from all planes depends on the respective intensities and thus residue Y would appear negative in the final spectrum.

It is realized from the above discussion that the C^{α}–C^{β} coupling evolution during the 2τ period is extremely crucial in determining the signs of certain peaks in the spectrum. In other words by manipulating this particular evolution it would be possible to alter the signs of the peaks at will. In this context, the 180° pulse (encircled in Fig. 1) on carbon channel, which serves the purpose of (i) refocusing C^{α} chemical shift and (ii) keeping active evolutions under C^{α}–C^{β} and C^{α}–N couplings, plays a crucial role. Three different possibilities, which are dictated by the ranges and distinctiveness of the C^{α} and C^{β} chemical shifts of the different residue types, can be conceived:

(i) In case of glycines, there is no C^{β} and thus the term E_1 would be unity (Eq. (5)), consequently, glycines are expected to appear oppositely phased with respect to the rest of the residues. However, additional complexities arise because of the fact that the ^1H–^{15}N correlation peak in the HSQC spectrum for glycine is a 'doublet of a doublet' along the proton dimension, since the amide proton is coupled to two H^{α} protons. In contrast, all other correlation peaks will only be doublets. As a result, the self and the sequential peaks originating from glycine H^{α} have different fine structures. Similarly, if there is a glycine at $i+1$ position, the self and the sequential peaks of the ith residue will have different fine structures. The glycine multiplet spans a greater width along the proton dimension. Additionally, since the glycines have two H^{α} protons, each one of them contributes independently to the self and the sequential peaks. Thus, the co-addition of peaks originating from glycine and other residues is not very simple and the final pattern is hard to predict for the self-peaks of glycines.

(ii) The C^{β} chemical shift of alanines is very distinct (18–20 ppm). The 180° pulse in the middle of 2τ period (encircled pulse in Fig. 1) can be made band-selective not to invert the C^{β} of alanines. This will eliminate the C^{α}–C^{β} coupling evolution of alanines, making E_1 term (Eq. (5)) unity for these residues, and thus after a phase shift of 180° in the $F_2(H^N)$ dimension for reasons described in the previous paragraphs, the alanine peaks will appear negative in the spectrum.

(iii) The C^{β} chemical shifts of serines and threonines are most downfield (60–79 ppm) compared to all other residues (18–43 ppm). A band-selective 180° pulse, in the middle of 2τ period (encircled pulse in Fig. 1), can be applied to selectively refocus all the non-glycine C^{α} carbons in the range 49–79 ppm and this would avoid the C^{α}–C^{β} coupling evolution for all the residues except for serines and threonines—for which the C^{β} carbons (60–79 ppm) also get inverted and C^{α}–C^{β} coupling evolves in a normal manner. Thus, the term E_1 becomes unity for all non-serine/threonine residues and remains negative for serines and threonines. This results in negative peaks for serines and threonines in the spectrum. It is important to note here that any attempt to include C^{α} carbons of glycines results in inversion of several C^{β} carbons as well.

Additionally, in the (HC)NH experiment, side chain amide proton correlation peaks are not observed and thus HSQC spectrum gets further simplified.

2.2. Application to proteins

The (HC)NH pulse sequence has been successfully tested on a 151 residue long calmodulin-like calcium-binding protein from the protozoan parasite *Entamoeba histolytica* (*Eh*CaM). The assignment of this protein has already been published [11] and thus this served as an excellent test case. Fig. 4 displays a section of the (HC)NH spectrum recorded on *Eh*CaM protein with different selective 180° pulses (encircled pulse in Fig. 1): (B) band-selective pulse of width 325 μs, covering 12,000 Hz (26–86 ppm) on carbon channel with offset at 56 ppm, and (C) band-selective pulse of width 650 μs, covering 6000 Hz (49–79 ppm) on carbon channel with offset at 64 ppm. Fig. 4A is the reference HSQC spectrum. In Fig. 4B, all the alanines appeared negative (red peaks) and are thus easily identified in the (HC)NH spectrum. To emphasize the same, alanines are marked with their respective numbers in the sequence. The position corresponding to Ala 106 is encircled in dash to indicate that this peak does not appear at this contour threshold. Similarly, in Fig. 4C, all the serines and threonines appeared negative

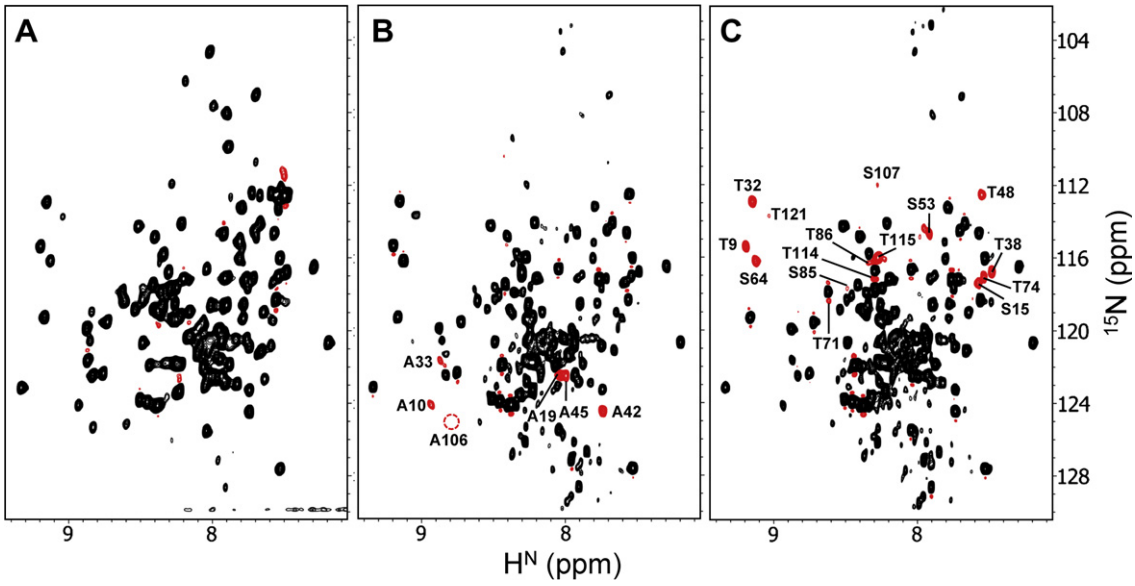

Fig. 4. Illustrative region of (HC)NH spectra for calmodulin-like Ca^{2+}-binding protein from the protozoan parasite *Entamoeba histolytica* (*Eh*CaM) (holo form; folded) in Tris (20 mM)–NaCl (100 mM) buffer (pH 7.0) with different selective 180° pulse (encircled pulse, Fig. 1) used in two different experiments: (A) Standard HSQC spectrum of the protein as reference. The red peaks here are processing artifacts (wiggles). (B) (HC)NH spectrum with a band selective pulse of width 325 μs (at 47.72 W), covering 26–86 ppm; C^{α}–C^{β} couplings do not evolve for alanines making those peaks appear negative in the 2D spectrum (after a 180° phase shift in $F_2(H^N)$ dimension); all the alanines have been marked here with their sequence positions; A106 position is encircled in dash to emphasize that this peak is not present at this contour level. (C) (HC)NH spectrum with a band selective pulse of width 650 μs (at 11.93 W), covering 49–79 ppm; C^{α}–C^{β} couplings do not evolve for any residue except for serines and threonines, and thus serines and threonines appear negative in the 2D spectrum.

(red peaks) and thus could be readily distinguished from the other residues in the HSQC spectrum. It is important to note here that there is no distinction among the serines and threonines, and both of them appear negative simultaneously. All the serines and threonines have been marked as per the sequence in the spectrum (Fig. 4C).

These ideas can easily be extended to produce an HSQC spectrum containing peaks corresponding to only alanines or serine–threonines. For this an experiment is recorded using a 180° pulse (encircled pulse, Fig. 1) which covers the full C^{α}–C^{β} chemical shift range (10–80 ppm) and the other experimental parameters are kept the same as for the experiment with the selective pulse. In this case, all the peaks would be of same sign and are chosen to be positive. Then, this data is subtracted in the time domain itself from the one recorded as in Fig. 4B or 4C, and the resultant after 2D Fourier transformation results in an HSQC spectrum containing peaks corresponding to alanines or serine–threonines only. Fig. 5 shows an example of such an experiment, recorded on calmodulin-like Ca^{2+}-binding protein from the protozoan parasite *E. histolytica* (EhCaM) (holo form; folded) in Tris (20 mM)–NaCl (100 mM) buffer (pH 7.0), where only serines and threonines have been picked up. This spectrum is equally informative as that in Fig. 4C, but without the complexities because of non-serine/threonine peaks. Additionally, any overlap of serine–threonine with peaks of other residues will also be filtered out in this spectrum. Similar filtering of alanines can also be obtained.

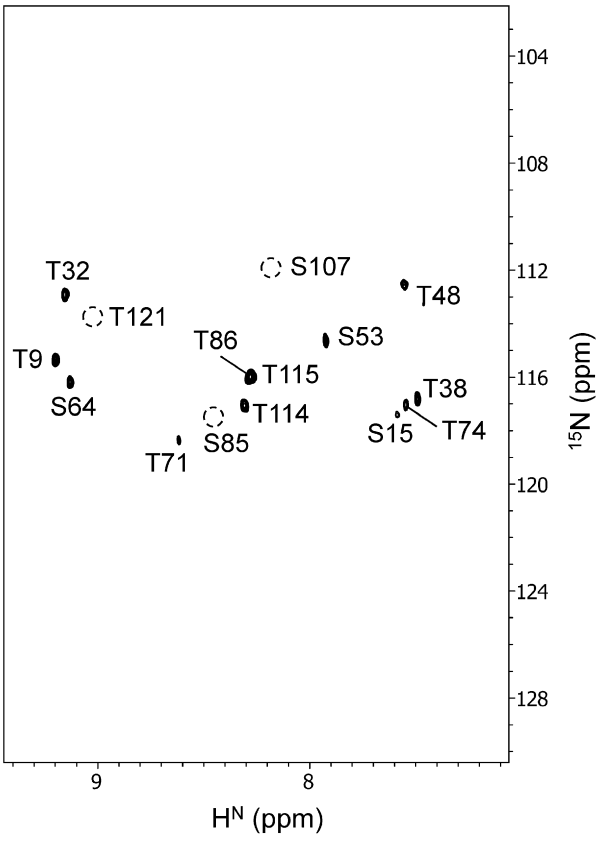

Fig. 5. Illustrative region of (HC)NH difference spectrum for calmodulin-like Ca^{2+}-binding protein from the protozoan parasite *Entamoeba histolytica* (EhCaM) (holo form; folded) in Tris (20 mM)–NaCl (100 mM) buffer (pH 7.0), where only serines and threonines are selected in the pulse-sequence scheme as explained in the text. Peak positions corresponding to S85, S107 and T121 have been encircled in dash to emphasize that these peaks are not present at this contour threshold.

2.3. Potential applications of (HC)NH

(i) The complete structural characterization of large proteins is till date a very challenging task. Several strategies are employed and complete deuteration is often used to sharpen the lines and thus improve the sensitivity and resolution in the spectra. In such a situation the present method which relies on the availability of H^{α} becomes inapplicable. However, large proteins can be studied with different view points where only a few residues and their behaviors can be looked at very selectively under specific set of conditions. In such a situation, the present pulse sequence can be made applicable by employing partial deuteration (say 50%), and the sensitivity can be enhanced by incorporating the TROSY strategy [12]. Higher signal averaging is affordable as this is only a 2D experiment. Thus one should be able to identify alanines and serine–threonines in the HSQC spectrum, even for a large protein. The specific assignments of the alanines and serines/threonines can be obtained by making several mutations in the protein, one at a time, at the locations of alanines or serine–threonines, and recording the (HC)NH spectra. This could then be used to monitor various changes along the protein sequence for a variety of purposes; for example, due to ligand binding, or due to changes in experimental conditions, or during protein folding studies, phosphorylation assays, etc.

(ii) This method helps to filter out peaks belonging to alanines and serines/threonines in the HSQC spectrum even when their peaks overlap with those of other residues, by performing a difference experiment (Fig. 5). Thus, even while working with smaller proteins some ambiguities can be resolved.

(iii) A natural extension of the above ideas is to introduce alanines or serine–threonines at various sites in a protein by site directed mutagenesis, assign them as described, and then probe their environments and characteristics using standard NMR methods in a more quantitative manner. This is akin to the strategy used in fluorescence studies wherein tryptophan residues are introduced at chosen sites for monitoring the local environments, protein folding pathways, etc.

3. Experimental

3.1. Protein preparation

^{15}N–^{13}C labeled holo *Eh*CaM was obtained as a kind gift from Mr. Ashok Kumar Rout. The protein expression, purification, and assignment have been described elsewhere [11].

3.2. NMR spectroscopy

All NMR experiments were performed at 25 °C on a Bruker Avance spectrometer equipped with a CryoProbe, operating at ^1H frequency of 800 MHz. The delays $2T_N$ and 2τ were set to 30 and 24 ms, respectively. Thirty-two complex points were used in the $F_1(^{15}N)$ dimension and 1024 complex points were collected in the $F_2(^1H)$ direct dimension. Sixty four scans were collected for each FID. The acquisition time for each experiment was approximately 3 h. A band selective $C^{\alpha/\beta}$ inversion pulse was applied using a standard Gaussian cascade Q3 pulse [13]. For serine–threonine labeling, the pulse width of 650 μs (at 11.93 W, inversion bandwidth ~6000 Hz) was used with carrier for the selective pulse on carbon channel was placed at 64 ppm; and for alanine labeling the pulse width of 325 μs (at 47.72 W, inversion bandwidth ~12,000 Hz) was used with carrier for the selective pulse on carbon channel was placed at 56 ppm. The carbon carrier frequency for all other carbon channel pulses was set to 56 ppm.

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